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Germline TP53 Mutations do not Always Cause Li-Fraumeni Syndrome

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Abstract

Background: *Li-Fraumeni syndromes are rare autosomal dominant disorders known to be associated with inherited TP53 mutations. In early onset breast cancer about 2% - 3% of germline mutations are contributed by TP53 gene whereas in other types of cancer it is 17%. Due to the low frequency (<1%) of TP53 mutations in hereditary cancers its mutations were rarely investigated. The present study focuses on the TP53 mutation pattern in Li- Fraumeni syndrome and in other hereditary cancer syndromes.*

Methods*: A total of 525 families were included in this study, 6 of which were Li-Fraumeni/Li-Fraumeni like (LFS/LFL) cases and for whom, the TP53 mutation analysis was done using PCR followed by Sanger sequencing technique. In the rest of the 519 hereditary cancer cases, the TP53 mutation was detected by targeted resequencing using Illumina Next generation sequencing platform.*

Results: *Three of the 6 patients with features suggestive of LFS/LFL had deleterious missense mutations. Of the 519 suspected hereditary cancer cases 10 had deleterious germline mutations in TP53 gene. R175H, R306* and W146* were found to be associated with poorer survival. Survival analysis for LFS/LFL cases did not show any difference in disease free and overall survival in the patients with and without deleterious mutations in TP53.*

Conclusions: *In conclusion, germline TP53 mutations can be seen in other types of cancers also and is not restricted to LFS/LFL alone.*

Key words*: Li-Fraumeni syndrome, TP53, Mutation, Cancer; Germline; targeted resequencing*

Introduction

TP53, a tumour suppressor gene is most frequently altered in germline in Li-Fraumeni syndrome (LFS) and as a somatic mutation in many cancers. Though the alterations have been found in every region of the protein (Leroy et al, 2013), frequently occurring mutations where only studied in depth for their contribution to cancer development.

LFS and Li-Fraumeni like syndrome (LFL) are rare autosomal dominant disorders known to be associated with inherited TP53 mutations (Eeles, 1995). The characterization of the LFS is based on Chompret's criteria. By the age of 30 the risk for an individual with LFS to develop any invasive cancer is ~50% and by age 70 it is 90%. Individuals with LFS are prone to develop second, third, even fourth malignancy (Malkin et al, 1990). Germline TP53 mutations contribute to 2% – 3% of early onset breast cancer (Lalloo et al, 2006) whereas in some of the cancers associated with LiFrauemni sites, TP53 mutation may contribute to up to 17% (Gonzalez et al, 2009). There are some rarer cancers associated with TP53 germline mutation which include choroid plexus carcinoma or papilloma before the age of 15, Wilms' tumor, and malignant phyllodes tumors (Birch et al, 2001; Gonzalez et al, 2009).

The mutation spectrum of the TP53 differs among cancers of the colon, lung, oesophagus, breast, liver, brain, reticulo-endothelial tissues, and hemopoietic tissues (Hollstein et al, 1991). In most of the studies wherein the whole coding sequence of the TP53 has been examined, about 86% of mutations cluster between codons 125 and 300 and with most of them being missense (87.9%), corresponding mainly to the DNA binding domain. In contrast, outside this region, missense mutations represent only about 40%, and the majority of mutations seen are either nonsense or frameshifts (Jones et al, 1992). The TP53 protein level is low or undetectable in normal cells, but diverse forms of stress may trigger its production, resulting in either cell cycle arrest or apoptotic cell death (Levine, 1997). Mutations in the TP53 gene lead to a version of p53 protein that cannot regulate cell growth and division effectively. Specifically, the altered protein is unable to trigger apoptosis in cells with mutated or damaged DNA. As a result, DNA damage can accumulate in cells; such cells may continue to divide in an uncontrolled way, leading to the growth of tumors. A dominant negative form of the mutant protein can bind to the normal p53 protein from the unmutated allele and lead to loss of function (Willis et al, 2004). Any loss or gain of function in TP53 gene due to mutation leads to an uncontrolled cell proliferation and cancer (George, 2011). Mutations in this gene may also regulate the miRNA expression.

Missense mutation in TP53 inhibits the expression of miRNA by its gain of function activity and thereby regulates the miRNA biogenesis in cancer (Gurtner et al, 2016).

A study by Soussi and Beroud [2001] showed TP53 protein function to be lost in almost 50% of human cancers and these mutations are believed to be associated with the initiation and progression of malignancies (Soussi and Beroud, 2001). Germline mutations in one TP53 allele are highly susceptible to cancer by inactivating the function of the gene in humans and in mice. They develop cancer very early in life and at very high frequencies (Malkin et al, 1990, Donehower et al, 1992). In addition, naturally occurring genetic polymorphisms in TP53 may also play a role in the development of Human Papilloma Virus (HPV) associated cancers (Storey et al, 1998).

Germ line TP53 mutation has been reported rarely in hereditary breast cancer (HBC), hereditary breast and/or ovarian cancer syndromes (HBOC) and hereditary non-polyposis colorectal cancer (HNPCC) or Lynch Syndrome, since in most of the HBC/HBOC cases BRCA1/BRCA2 and in HNPCC, hMLH1, hMSH2, hMSH6, PMS1 and PMS2 genes were only investigated. Due to the low frequency (<1%) of TP53 mutations in HBC/HBOC/HNPCC families, its mutations were rarely investigated (Lalloo and Evans, 2012). In addition, in the past, the mutation screening was based on heteroduplex detection by denaturing high-performance liquid chromatograpy (DHPLC), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single-strand conformation polymorphism(SSCP), conformation-sensitive gel electrophoresis (CSGE) and enhanced mismatch mutation analysis (EMMA) which were labour intensive.

Recently, Next Generation Sequencing (NGS) platform has been used for screening a panel of genes (targeted re-sequencing) and reduces the time and cost. In this study we have performed Targeted resequencing by Illumina next generation sequencing platform. Illumina's sequencing by synthesis (SBS) technology is the most successful and widely adopted next-generation sequencing (NGS) technology worldwide. Illumina sequencing instruments and reagents support massively parallel sequencing using a proprietary method that detects single bases as they are incorporated into growing DNA strands. The SBS chemistry provides us the accurate data across the broad range of application (Illumina Inc., Singapore).Thus the present study focuses on the TP53 germline mutation pattern in Li- Fraumeni syndrome and in other hereditary cancer syndromes.

Study Design

Patient Samples: 10ml of peripheral blood sample was collected from the patient's attending the Hereditary Cancer Clinic, after getting the family history details and the patient's informed consent. The blood sample from the patient was taken only if he/she satisfied the eligibility criteria (Rajkumar et al.,2003) (Supplementary Table S1) . For Li-Fraumeni cases, the inclusion criteria was based on Chompret's criteria (N=8) and for rectal cancer cases, we followed Bethesda Criteria (Rajkumar et al., 2000).

A total of 525 families were included in this study, of which for 6 Li-Fraumeni (LFS/LFL) cases the TP53 mutation analysis was done using PCR followed by Sanger sequencing technique covering exons 2 to 11 while for the rest of the 519 hereditary cancer cases the TP53 mutation was detected by targeted resequencing of 30 genes (Rajkumar et al, 2015) and later 56 genes using Illumina Next generation sequencing platform (Supplementary Table S2).

PCR & Sequencing: DNA was extracted from peripheral blood mononuclear cells using the Qiagen's QIAmp DNA blood kit, as per the manufacturer's protocol. 100ng of the DNA was used to amplify the entire coding region of the TP53 gene in a 25 µl reaction. The primer sequences used to amplify exons 5-8 were as published by Vet et al, 1994 and for exon 2-4 and 9-11 were as published by Verselis et al, 2000. The amplified PCR products were directly sequenced in both the directions using Big Dye terminator kit v3.1 (Applied Biosystems by Life technologies, Singapore) according to manufacturer's instructions. The final sequencing PCR product was run in ABI PRISM 310 Genetic analyzer and analysed using Sequence analysis v3.2.1 software.

Immunohistochemistry: Immuno-histochemistry (IHC) for p53 was done on 5□m representative sections of malignant phylloides tumour, oligo-astrocytoma of the brain and adenocarcinoma of the rectum (Figure 1 D, E, F) as described earlier using DO-7 antibody (Cooper and Haffajee, 1997).

Targeted resequencing: Briefly, 1µg of DNA was sheared using Bioruptor (Diagencode, Belgium) with a fragment size of 250bp. Then the library preparation was done by using Sureselect XT2 library preparation kit (Agilent Technologies, Inc, USA), as per the manufacturer's instructions. Using the custom capture library kit the hybridisation was performed which allows capture of <3Mb library size of the targeted sequences including 2kb upstream and downstream of the 56 genes. 8pmoles of the captured library DNA was denatured and subjected to cluster generation using cBOT instrument (Illumina Inc, Singapore), on a

paired end flow cell v.3.0. The sequencing was done using SBS version 3.0 reagents, in a HiScan SQ instrument (Illumina Inc, Singapore).

Data analysis: The .bcl files generated by the instrument were converted into FASTq files using Casava software (Illumina Inc, Singapore). CLC Bio suite (CLC Bio, Aarhus, Denmark) version 7.0 was used for further processing the indel data. Alignment was done using all the 24 chromosomes as reference from NCBI GRCH38.

Indels in coding region and SNP's resulting in premature termination and deleterious mutations based on the IARC TP53 Database (Bouaoum et al, 2016) were taken up for validation using Sanger sequencing.

Statistical analysis: The association between the deleterious mutations with the clinic-pathological variables for LFS cases were analyzed using chi square test. Survival analysis for LFS cases was done using the SPSS v13 software.

Results

PCR followed by the Sanger sequencing for the 6 LFS/LFL patients showed three missense mutations with deleterious effect (I255S, R337C and R342P). The rest of the 3 did not show any change in the sequenced regions of the TP53 gene. Of the 3 deleterious mutations, Ile255Ser is a germline mutation detected in a 21years old female diagnosed to have multiple cancers i.e., endodermal sinus tumour of right ovary diagnosed at 14 years, Malignant phylloides tumor of Right Breast, adenocarcinoma of rectum and mixed oligo astrocytoma of brain all diagnosed at 21 years with no history of cancer in the family. The patient's parents were negative for this mutation, suggesting a de novo germline mutation. The Immunohistochemistry for the TP53 protein in breast and brain tissue showed 25-50% positivity and the rectal tumor tissue showed >50% positivity (Figure 1). We have performed the sequencing in all the three tissue samples and found the loss of heterozygosity (LOH) for I255S in breast and brain tissue samples. The rectal tissue was heterozygous for the mutation probably due to higher stromal component than the tumour cell content.

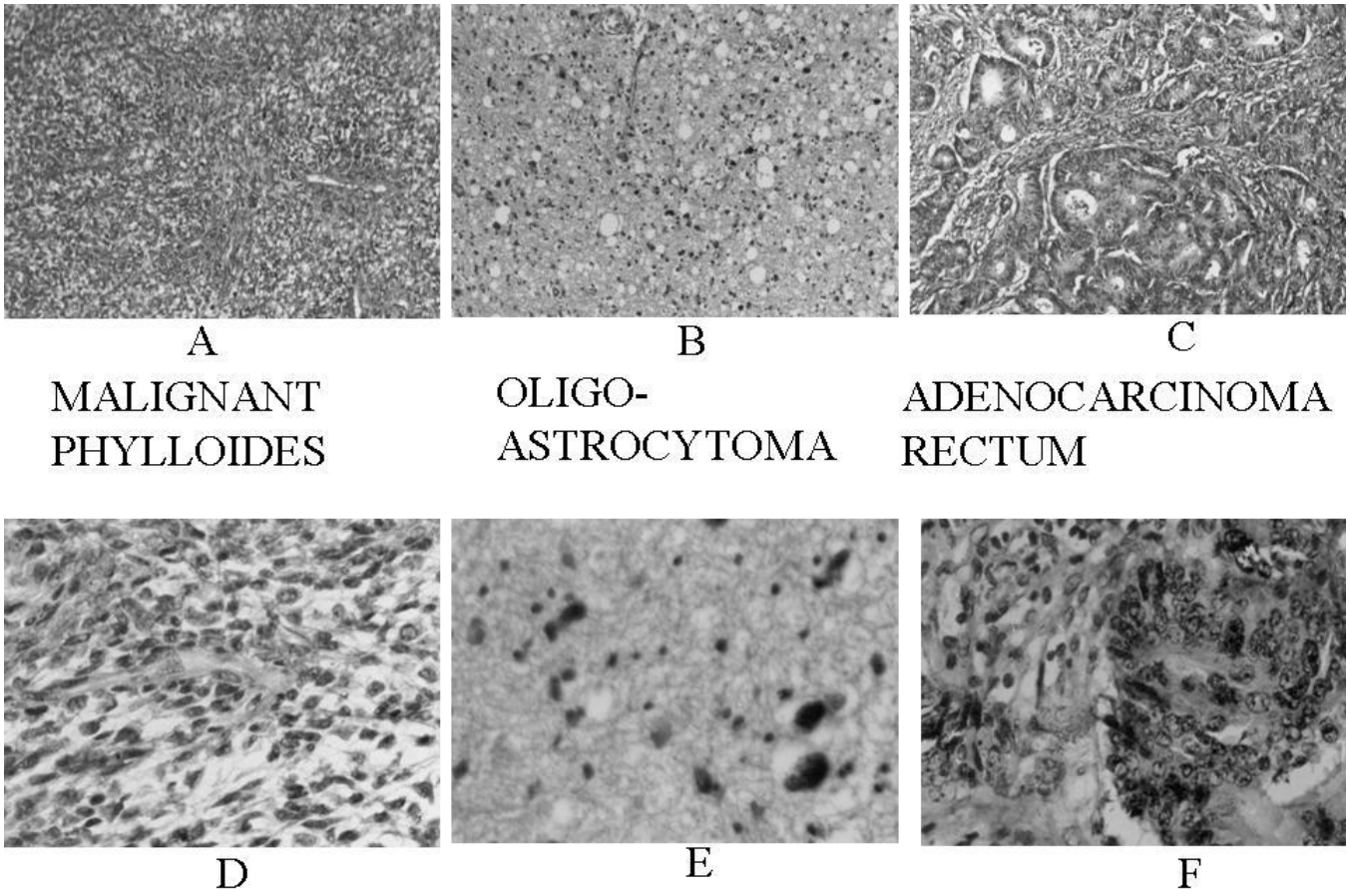

Figure 01 A, B, C: H &E staining of the breast malignant phylloides tumour, brain mixed oligo astrocytoma tumour and adenocarcinoma of the rectum, respectively. (magnification x 10).

D, E, F: Immunohistochemistry for p53 expression in the breast malignant phylloides tumour, brain mixed oligo astrocytoma tumour and adenocarcinoma of the rectum, respectively. (magnification x 40).

R337C and R342P missense mutations are categorized as deleterious mutations in IARC TP53 database (Bouaoum et al, 2016) and were seen in two cases. R337C was seen in a male proband with skin cancer detected at the age of 28 years with the family history of two first degree relative with LFS spectrum of cancers. R342P was seen in a patient diagnosed to have breast cancer, soft tissue sarcoma and chest wall tumour at the age of 41 years with the family history of LFS spectrum. The clinical details of the patients are given in the Table 1. For the 3 LFS cases who did not show a deleterious TP53 mutation, CHEK2* 1100delC mutation screening was done and was found to be negative for the mutation.

NGS analysis for 519 hereditary cancer cases showed 10 mutations in TP53 gene categorized as deleterious mutations in IARC TP53 database including R248Q, N263D, G245S, R273H, R273C, R175H,

C242R, R267Q, R306* and W146*. Of the 10 mutations, R248Q was seen in two cases with Li-Fraumeni syndrome. The mutations detected by NGS analysis were all further validated by Sanger sequencing. The clinical details including age, site of cancer and family history details are given in Table 2. The frequency of TP53 gene mutation in LFS/LFL and other hereditary cancers are shown in the figure 2.

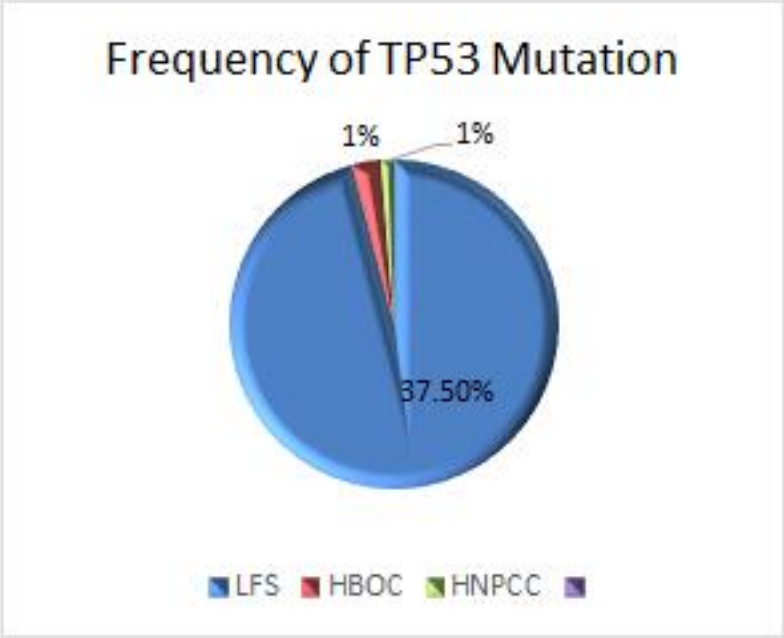

Figure 02 Frequency of TP53 gene mutation

The survival analysis for disease free and overall survival based on the TP53 deleterious mutation status in LFS/LFL did not show any statistical significance.

S.NO	HCD No	TP53 Mutation	Age/Sex	Site of Cancer	F/H	PATHOGENIC
1	96/04	c.764T>G; p.I255S	21/F	multiple cancers - germ cell ovarian tumour, malignant phyllodes tumor Bst, Ca rectum, Astrocytoma of brain	Nil	YES [IARC DATABASE]
2	102/04	c.1009 C>T; p.R337C	28/M	Skin Cancer	Brother- OsteoSarcoma, Mother- Ca breast, Sister- Ca Lung	YES [IARC DATABASE]

3	17/05	c.1025G>C ; p.R342P	41/F	Ca Breast & Soft tissue sarcoma	2 sisters- Ca brain, daughter- Osteosarcoma, Mother- Ca breast, Maternal Uncle - Ca brain	YES [IARC DATABASE]
4	199/05	No Mutation	20/F	Ca Right Breast	Sister-blood cancer	
5	68/06	No Mutation	29/F	Ca Breast	Brother- ALL, Maternal Aunt- Ca breast, Maternal Uncle- Ca lung	
6	95/06	No Mutation	35/F	Fibro adenoma of breast	Elder daughter- corpus collosum, 2nd daughter- ALL, *MGM- Ca stomach, *PGF- Ca stomach	
7	82/06	c.743 G>A; p.R248Q	41/F	Osteosarcoma & Ca right Breast	Nil	YES [IARC DATABASE]
8	68/07	c.743 G>A; p.R248Q	12/M	Acute Lymphoblastic Leukemia	Sister- Ca Kidney, Grandfather- Ca stomach, Paternal cousin- Ca lung	YES [IARC DATABASE]

MGM- Maternal grandmother; MGF- Maternal grandfather; PGF- paternal grandfather; Ca- cancer

Table: 1 Characteristics of TP53 mutation in Li-Fraumeni/Li-Fraumeni like syndrome cases

S.NO	HCD No	TP53 Mutation	Site of Cancer	F/H	Age/Sex	PATHOGENIC
1	8/03	c.787 A>G; p.N263D	Ca Right Breast	Nil	23/F	?PARTIALLY FUNCTIONAL [IARC DATABASE] LOW MDM2 TA ACTIVITY
2	3/07	c. 916 C>T; p.R306*	Ca Left Breast	Sister- Ca breast	41/F	YES [Chappuis P.O. *et al*., 1999]
3	64/06	c. 733 G>A; p.G245S	Ca Left Breast	*PGM– Ca breast, *PA– Ca breast	32/F	YES [IARC DATABASE]
4	24/09	c. 818 G>A; p.R273H	Ca Ovary	*MGF - ?Cancer	27/F	YES [IARC DATABASE]
5	1/06	c. 800 G>A; p.R267Q	Ca Ovary	Sister- Ca Breast	33/F	PARTIALLY FUNCTIONAL [IARC DATABASE

6	124/06	c.817C>T; p.R273C	Ca Rectum	Father- Ca Stomach	31/M	YES [IARC DATABASE]
7	48/08	c.524 G>A; p.R175H	Ca rectum & Ca Stomach	Father-?Ca	38/M	YES [IARC DATABASE]
8	53/14	c.724 T>C; p.C242R	Ca Rectum	Nil	19/M	YES [IARC DATABASE]
9	54/06	c.437G>A; p.W146*	Ca Left Breast	Father- Ca stomach *MGF- Ca anal canal	32/F	YES [IARC DATABASE]

** PGM- Paternal grandmother; PA-Paternal Aunt; MGF- Maternal grandfather; Ca-Cancer*

Table: 2 Characteristics of TP53 mutation identified among 524 hereditary cancer cases

Discussion

Of the eight patients with LFS/LFL features, 5 [62%] were found to carry a germline deleterious mutation in TP53 gene. In general, over 50% of the LFS cases have identifiable germline mutation in TP53 gene (Nicholes et al, 2001).

The mutation I255S falls in the DNA binding domain region of the TP53 gene, considered to be the hot spot site for the TP53 mutations (Whibley et al, 2010). The physico-chemical property of the wild type amino acid Isoleucine's hydrophobic nature was changed to Serine's polar nature. Since the mutation falls in the DNA binding region of the domain, it affects cell cycle, apoptosis and some of the signalling pathways (Reles et al, 2001). In most of the studies, the Isoleucine amino acid at codon 255 is mutated into Asparagine or Phenylalanine which leads to a conformational change in TP53 protein (Hollstein et al, 1994; Trivers et al, 1995 and Brandt-Rauf et al, 1996). Interestingly, while p.I255S, p.I255F, p.I255N, p.I255T have been reported to have a loss of Transcriptional activity and reported in tumours as somatic mutation, till date none of them have been seen as a germline mutation. Our report on I125S will therefore be a novel germline TP53 mutation. Additionally, the LOH of the gene mutation was confirmed in the tumour tissues of the breast and brain.

R337C and R342P are two deleterious mutations falling in the tetramerization domain region of the TP53 gene and these mutations exhibit an overall decrease in DNA-binding and transactivation activity (Davison et al, 1998; Lomax et al, 1998; Atz et al, 2000 and Johnson et al, 1995). A study by Greenblatt et al (1994) observed that the R337C mutation occurs at a CpG dimer site which is frequently found as a

germ line mutation. R342P, a substitution was found to compromise both the transcriptional activity and tetramerization of the TP53 gene. These mutations in TP53 gene may predispose to multiple cancers in LFS/LFL syndrome. (Fiszer-Maliszewska et al, 2009, Palmero et al, 2008).

Our data using NGS showed 11 deleterious mutations: 2 in LFS/LFL syndrome; 6/346 in HBC/HBOC and 3/45 in early onset rectal cancer cases. Of the nine hereditary cancer cases, eight (88%) of them were early-onset cancer cases diagnosed below 35yrs of age and they do not meet the criteria for LFS. R175H, C242R, G245S, R248Q, R273H, R273C, N263D and R267Q mutations falls in the central DNA binding domain region of the TP53 protein and are known to destabilise the structural property of TP53 gene by inducing a conformational change in DNA binding surface (Olivier et al, 2010). All have also been reported as germline and somatic mutations in the IARC database (Bouaoun et al, 2016). A study by Martin et al, (2003) with TP53 G245S mutation in breast cancer family concluded that germline TP53 mutations are not an important cause of multiple primary cancers outside the setting of LFS which is relevant to our data. The germline mutations in TP53 are rarely associated with the presence of multiple primary cancers in breast cancer families and support previous studies suggesting that TP53 mutations account for less than 1% of hereditary susceptibility to breast cancer. The mutation R248Q was found in a wide range of cancers and causes gain of function activity in breast cancer and lung cancer (Shtraizent et al, 2015 and Yoshikawa et al, 2010). A molecular dynamic simulation study by Ng et al., (2015) in TP53 DNA binding domain with R248Q results in a conformational change and inablity to interact with the major groove of the DNA. The mutation R273H has a deleterious effect because it stimulates the IGF-1R-AKT pathway by suppressing the miRNA mir-30a in breast cancer (Guo et al, 2016).

The mutations R175H, C242R, R273C and R267Q were found to be deleterious and we have seen these mutations in four early onset cancer cases. The patient with R175H mutation had a poor outcome which was similar to the study by Goh et al (1995) who showed that the TP53 codon 175 mutation contributes to an aggressive disease and a poor survival in colon cancer.

Conclusion

It is proven that the deleterious mutation in the TP53 gene has the functional consequence as it compromise the activity of the gene in the DNA repair mechanism. Individuals with germline TP53 mutation are at risk of developing cancer much earlier in life and it is also possible that the interaction with other factors with TP53 gene can contribute towards cancer risk.

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